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Research paper

Effects of dietary arachidonic acid on the reproductive physiology of female Atlantic cod (*Gadus morhua* L.)

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ABSTRACT

The present study was designed to investigate potential effects of arachidonic acid (ARA) on the reproductive physiology of female Atlantic cod (*Gadus morhua* L.).

Two-year old Atlantic cod of both sexes were equally distributed into eight sea cages after completion of their first spawning in May 2005. Four experimental groups were established and fed diets with different levels of ARA corresponding to 0.5, 1, 2 and 4% of total fatty acid. Ovarian growth and development was documented every month. Fatty acid composition was analysed in ovaries, liver and plasma at the beginning of the experiment, one month prior to spawning, and in spent fish, one month after spawning was completed. Plasma concentrations of estradiol-17β, testosterone and vitellogenin, and ovarian gene transcript levels of steroidogenic acute regulatory protein (*star*), P450aromatase (*cyp19a1a*) and 20β-hydroxy steroid dehydrogenase (*20bhsd*/*cbr1*) were monitored every month in fish fed the experimental diets and related to oocyte stage. Potential fecundity was calculated based on ovarian samples taken one month before onset of spawning.

Ovarian and plasma ARA levels were highly correlated to dietary ARA levels. There was a net accumulation of ARA compared to other essential fatty acids in ovarian tissue that was reflected in a decrease in EPA:ARA ratio. Plasma concentrations of vitellogenin, estradiol-17β and testosterone and key gene transcript levels were affected by dietary ARA and stage of maturation. The results show that ARA has a significant influence on the reproductive physiology of female Atlantic cod.

1. Introduction

The Atlantic cod (*Gadus morhua* L.) is one of the commercially most important species in northern European fisheries, and is a candidate species for aquaculture in northern Europe and Canada. While a decline in several wild cod stocks led to an increased interest in aquaculture in the late 1990s, there is also evidence that altered mean sea temperatures can lead to a change in the composition of the food web, changes in the distribution of fish species and hence a possible change in diet composition for the cod which is a top predator (Drinkwater, 2005; Røjbek et al., 2012; Rose, 2005). Broodstock diet is important both to maintain fish health and to ensure an optimal nutrient content of the yolk for the developing embryo. Thus, diet composition may have implications for both cultured and wild cod stocks.

Body composition, especially with regards to fat, has a profound influence on oocyte recruitment, fecundity and atresia, as has been documented in Atlantic cod, both in wild-captured fish and experimentally (Karlsen et al., 1995; Kjesbu and Holm, 1994; Kjesbu et al., 1991; Marshall et al., 1999; Skjaeraasen et al., 2010). Studies on broodstock nutrition in cod, as well as other fish species, generally focus on the effect of diet on fecundity, egg and larval viability and biochemical composition of the eggs (Lie et al., 1993; Silversand et al., 1995; Røjbek et al., 2014; Hemre et al., 1995; Mangor-Jensen et al., 1994; Pickova et al., 1997; Salze et al., 2005;Izquierdo et al., 2001; Furuita et al., 2003; Mazorra et al., 2003; Lanes et al., 2012). The essential, highly unsaturated fatty acids (HUFA) arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 20:6n-3) have received special attention (cf. Bell and Sargent, 2003; Tocher, 2010). ARA, in particular, has been extensively studied in relation to reproductive performance. ARA content was reported to be higher, and EPA:ARA ratio lower, in eggs and ovaries obtained

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from wild cod and other marine fish, such as Senegalese sole (*Solea senegalensis*), than in cultured broodstock. These factors were suggested to be related to higher viability in eggs from wild fish (Norambuena et al., 2012; Salze et al., 2005).

A diet supplemented with ARA concentrations higher than those obtained with northern hemisphere fish oils, can increase fecundity, egg quality and viability of larvae in marine teleosts such as European sea bass (*Dicentrarchus labrax*), Japanese flounder (*Paralichthys olivaceus*), Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic cod (Bruce et al., 1999; Furuita et al., 2003; Mazorra et al., 2003; Røjbek et al., 2014). ARA is the precursor of biologically active eicosanoids, such as prostaglandins and leukotrienes, that are involved in many aspects of reproduction (e.g. Goetz and Garczynski, 1997; Kobayashi et al., 2002; Mercure and Van Der Kraak, 1996; Stacey and Goetz, 1982; Stocco et al., 2005). In addition, ARA can act directly on steroid biosynthesis, at least in mammals, as a regulator of the steroidogenic acute regulatory protein (StAR), which mediates transport of cholesterol across the mitochondrial membrane in a rate-limiting step in steroidogenesis (Stocco, 2001; Stocco et al., 2005; Wang et al., 2000). Available evidence also suggests a role for StAR in steroidogenesis in fish, although the structure and regulation of the StAR protein may differ from mammals (e.g. Nunez and Evans, 2007). EPA and ARA compete for the enzymatic pathways involved in eicosanoid synthesis. The resulting eicosanoids may, however, exert different or even opposite actions on reproductive processes, where those derived from ARA are generally more active (Mercure and Van Der Kraak, 1995; Sorbera et al., 2001; Wade et al., 1994).

In Atlantic cod in Norwegian waters, vitellogenesis normally starts in October, while the peak in spawning activity occurs in February–March (Kjesbu, 1994). Cod eggs are small, and vitellogenesis takes place in the months immediately prior to spawning, as well as during the spawning period (Kjesbu et al., 1991, 1996a,b). The Atlantic cod is a periodic spawner, and each female can release 15–20 batches of pelagic eggs at 50–100 h intervals, during a period of 3–4 weeks (Kjesbu, 1989). The early stages in oogenesis, as well as vitellogenin synthesis, are stimulated by estradiol-17β (E2), which is the major estrogen in teleost fish and is synthesized from testosterone (T) in the granulosa cells of the ovarian follicle. Conversion of T into E2 is catalysed by an enzyme complex containing ovarian P450c17-I, P450 aromatase (product of the *cyp19a1a* gene), and a flavoprotein NADPH-cytochrome P450 reductase (Simpson et al., 1994). Changes in *cyp19a1a* gene expression and P450aromatase enzyme activity are major regulators of ovarian production of E2 during reproduction and development (Chang et al., 1997). The ARA derived prostaglandin PGE2 stimulates ovarian synthesis of E2 in mammals, and ARA stimulates synthesis of testosterone (T) and E2 *in vitro* in goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*) ovarian follicles (Abayasekara and Wathes, 1999; Lister and Van Der Kraak, 2008; Mercure and Van Der Kraak, 1996; Van Der Kraak and Chang, 1990).

During final oocyte maturation, a shift in steroidogenesis occurs in the follicles, leading to the production of maturation-inducing steroid (MIS), instead of conversion of T into E2. This shift is mediated by a down-regulation of ovarian P450c17-I and P450aromatase, and an up-regulation of P450c17-II and 20β-dehydroxysteroid dehydrogenase (20β-HSD; encoded by the *20bhsd* or *cbr1* gene) (cf. Nagahama and Yamashita, 2008). The MIS is produced by the action of 20β-HSD (Simpson et al., 1994) and has been suggested to be 17,20β,21-trihydroxypregn-4-en-3-one (17,20β,21-P) in cod (Tveiten et al., 2010). It is well documented that ARA-derived eicosanoids are involved in regulation of final maturation and ovulation, in both fish and mammals (Abayasekara and Wathes, 1999; Goetz and Garczynski, 1997; Lister and Van Der Kraak, 2008; Mattos et al., 2000; Patino et al., 2003; Wathes et al., 2007; Sorbera et al., 2001; Knight and van Der Kraak, 2015). A surge of MIS just before ovulation stimulates synthesis of eicosanoids, which in turn activate the mechanisms involved in ovulation (Goetz and Garczynski, 1997; Patino et al., 2003).

While the actions of ARA *in vitro* have been extensively studied, and the influence of fatty acids in general on realized fecundity and egg and larval quality has received much attention, there is relatively limited knowledge on the effects of ARA and other HUFAs on fish reproductive physiology *in vivo* (e.g. Carrillo et al., 1995; Cerda et al., 1995; Navas et al., 1998; Norambuena et al., 2013a; Xu et al., 2017). The objective of the present study was to investigate effects of dietary ARA on the molecular and endocrine regulation of oogenesis in a commercially important, coldwater marine teleost, the Atlantic cod, in order to gain further insight into the physiological actions of ARA in fish in general and in this species in particular. ARA, DHA and EPA were monitored in plasma, liver and ovaries of female cod given four different dietary ratios of ARA in relation to total fatty acids. Plasma concentrations of the sex steroids E2 and T, and the yolk protein precursor vitellogenin (VTG) were measured as markers for sexual maturation, and gene transcript levels of *star* and the key enzymes *cyp19a1a* and *20bhsd* were chosen as markers for steroidogenesis.

2. Materials and methods

2.1. Diets

Four isolipidic and isonitrogenous diets were produced as extruded 9 mm pellets by Skretting ARC (Stavanger, Norway). Dietary composition and proximate analyses are given in Supplement S1. A regression design with stepwise increases in dietary ARA levels was chosen. The targeted gradient was achieved by exchanging fish oil with Vedovar (35% arachidonic acid, DSM Food Specialties, Delft, The Netherlands) to obtain 0.5, 1, 2 and 4% ARA of total fatty acids (FA) in the diets, respectively. The fish oil was of Scandinavian origin, and was rich in EPA and DHA and low in ARA. The ARA content in the diets was assumed to be within the physiological range. There was a decrease in EPA + DHA contents (1.8% of total FA) and monoenes (3.4% of total FA) in the diets with increasing levels of ARA (Supplement S2).

2.2. Nutrient analyses in diets

Feeds were sampled at time of production and analysed for proximate composition and fatty acids at Skretting ARC. Dry matter was determined by differences in weight after drying at 104 °C for 24 h. Total nitrogen was determined using the Kjeldahl method and crude protein calculated as N × 6.25. Fat was determined gravimetrically after acid hydrolysis and extraction with di-ethyl ether and ash gravimetrically after combustion at 540 °C for 16 h. Fatty acid profiles of the diets, were analysed after methylation of the fatty acids in methanolic HCl and extraction in hexane. The methyl esters were injected automatically on a gas chromatograph (Perkin Elmer Autosystem GC equipped with a programmable Split/Splitless injector, a CP Wax 52 column (L = 25 m. ID = 0.25 mm. df = 0.20 μm), a flame ionisation detector, He as carrier gas and a 1022 data system.

2.3. Feeding experiment

The experiment was performed at the Institute of Marine Research (IMR), Austevoll Research Station (60°N), from May 2004 to May 2006. One-year-old Atlantic cod (Norwegian Coastal Cod, n = 3200; average weight = 840 g), hatched and first-fed in a semi-intensive production system at the IMR production pond at

Parisvatnet, were kept in one sea cage (20 × 20 m) and fed a standard commercial cod diet (Skretting, Stavanger, Norway) from August 2004 until May 2005, through their first reproductive season. In May 2005, the fish were divided into four groups (n = 600) and stocked into 8 sea cages (5 × 5 m) with 300 fish (average weight: 2620 g) per cage. Experimental diets were fed by automatic feeders to duplicate groups of fish up to the beginning of the next spawning season, in January 2006. Feeding was stopped during spawning.

2.4. Sampling

Female cod were sacrificed each month from June 2005 to May 2006 (n = 5 in each diet group from June to October; n = 10–20 in each diet group from November to May). Individual fish were euthanized in an overdose of MS222 (200 mg.l^{-1}), weighed to the nearest g and measured to the nearest cm. Ovaries were dissected out and gonadosomatic index (Ig) was calculated as 100 × ovary weight/(body weight − (liver weight + intestine weight)). Blood was withdrawn from the caudal vein with a cold, heparinized syringe and plasma was separated by centrifugation for 3 min at 3000 rpm. After centrifugation, plasma was immediately frozen on dry ice. Ovarian and liver samples were dissected out and frozen on dry ice or snap-frozen in N2(l) for fatty acid analysis and molecular biology, respectively. Ovarian subsamples taken from the middle of the right ovary were fixed in 3.6% buffered formaldehyde (29.5 mM $NaH_2PO_4{\cdot}H_2O$ and 460 mM $Na_2HPO_4{\cdot}2H_2O$) for determination of potential fecundity. Frozen samples of plasma, liver and ovaries were stored at −80 °C until analysis.

2.5. Tissue fatty acid analysis

Fatty acid composition in plasma, ovaries and liver was analysed at NIFES using the method of Lie and Lambertsen (1991).

2.6. Ovarian stage classification

Ovarian samples were fixed for at least 14 days before measurements of oocyte size and calculation of potential fecundity according to Thorsen et al. (2006). Ovaries were classified according to the leading cohort (LC) oocyte size as pre-vitellogenic (PV), early vitellogenic VI, mid-vitellogenic (VII), late vitellogenic (VIII), spawning (S), with appearance of hyaline oocytes/eggs, or spent (SP), with residual eggs present in the ovarian cavity (Table 1). The different stages or steps are somewhat overlapping, as oocyte development is a dynamic process and it is difficult to identify the beginning or the end of each event (cf Lubzens et al., 2010). The classification of ovaries therefore must be seen in connection with time of sampling.

Table 1
Classification of ovaries of female Atlantic cod based on the appearance of oocytes and mean fresh Leading Cohort diameter (LC).

Ovarian stage		Oocyte appearance	LC (μm)
Stage PV	Previtellogenic	Transparent previtellogenic oocytes	<300
Stage VI	Early vitellogenic	Semi-transparent oocytes	−300–499
Stage VII	Mid-vitellogenic	Semi-transparent and opaque oocytes	−500–699
Stage VIII	Late vitellogenic	Opaque oocytes	−700–870
Stage S	Spawning*	Opaque and translucent (hyaline) oocytes	−705–940
Stage Sp	Spent**	Transparent previtellogenic oocytes	<300

* Spawning fish generally also had ovulated eggs >1100 μm present in the ovarian cavity.
** Shortly after the spawning season assuming all individuals had been active members of the spawning population.

2.7. Potential fecundity

Potential fecundity was estimated from pre-spawning ovarian samples using the Auto-diametric fecundity method (Thorsen and Kjesbu, 2001). A subsample was spread on a Petri dish and photographed with a resolution of 0.206 pixels/μm. The sizes of at least 200 vitellogenic oocytes were then analysed from the pictures by automatic particle analysis using the open source image analysis program ImageJ (http://imagej.nih.gov/ij/). From the mean oocyte diameter, oocyte density (n/g ovary) was estimated using a packing density formula given by Thorsen and Kjesbu (2001). Potential fecundity was then estimated by multiplication of oocyte density and ovary weight.

2.8. Analysis of plasma steroids and VTG

Steroids were extracted from plasma samples as described by Hyllner et al. (1994), dissolved in 1 ml of buffer (0.1 M phosphate pH 7.4, 0.4 M NaCl, 1 mM EDTA, 0.1% BSA) by heating (60 °C; 10 min) and stored at −20 °C until analysis by enzyme-linked immunosorbent assay (ELISA) (Cuisset et al., 1994; Rodriguez et al., 2000) validated for cod (Dahle et al., 2003). Extraction efficiencies were 95% for T and 94% for E2, respectively. Steroid standards were purchased from Sigma-Aldrich. Antisera, acetylcholine esterase labeled tracers and coated microtiter plates (Mouse-anti-Rabbit) were manufactured by Cayman Chemicals (USA). Details on cross-reactivity of the antisera against T and E2 are given by the manufacturer. The lower limits of detection were 10 pg/ml for T (ED80) and 15 pg/ml for E2 (ED90). The inter-assay coefficients of variation for the extracted biological reference plasma were 7,6% for T (n = 33) and 7,7% for E2 (n = 20). The intra-assay coefficients of variation were 5.6% for T and 6,8% in the E2 assay.

Plasma VTG was analysed by ELISA, with a slight modification of the method described by Meier et al. (2007). Briefly, VTG was isolated from plasma from E2-treated juvenile cod by anion exchange chromatography (Silversand et al., 1993). The concentration of the purified VTG was determined by UV absorbance spectrophotometry, using a coefficient of 0.66 corresponding to 1 mg/ml (Norberg and Haux, 1985). Standard solutions were prepared from the purified VTG solution at a concentration range of 40–0.0078 μg ml^{-1} in 0.01 M $KHPO_4$, 1% BSA (assay buffer). Plasma samples were diluted from 1:500 to 1:25,000, depending on VTG concentration. Standards in triplicate, and diluted plasma samples in duplicate were added to 96-well Maxisorp immunoplates (NUNC), coated overnight with 50 ng VTG in 0.02 M NaHCO3, pH 9.6. Polyclonal anti-cod VTG (Silversand et al., 1993) was added to the wells at a dilution of 1:100,000. The immunoplates were incubated at 4 °C overnight. The following day, plates were washed and incubated for 2 h at room temperature with goat-antirabbit secondary antibody conjugated with horseradish peroxidase (BioRad). The plates were again washed, TMB peroxidase EIA substrate (BioRad) was added to the wells and the plates were incubated for 2 h in the dark before the enzyme reaction was stopped by addition of 1 M H2SO4. Absorbance at 405 nm was read in a UVMax microplate reader (Molecular Devices).

2.9. RNA extraction and reverse transcription

Samples stored at −80 °C were cut into small pieces on dry ice before immediate transfer to cold Tri Reagent (Sigma–Aldrich). After homogenization in a FastPrep microfuge tube containing Lysing Matrix D ceramic beads, total RNA was extracted by the acid phenol–guanidinium thiocyanate method followed by purifying

the RNA through a column (RiboPure kit, Ambion/Applied Biosystems), and quantified by spectrophotometry. Ten μg of RNA were DNase treated (TurboDNA-free, Ambion/Applied Biosystems) in a 50 μl volume before the remaining RNA was again quantified by spectrophotometry. Random-primed cDNA was synthesized from exactly 500 ng DNase-treated RNA using a Reverse Transcription Core Kit (RT-RTCK-05, Eurogentec, Seraing, Belgium), and diluted 1:10 in nuclease-free H_2O before use in the quantitative real time PCR. RNA quantity and quality were determined in a representative number of samples, by capillary electrophoresis using the Lab on a Chip technique (Agilent 2100 BioAnalyzer, Santa Clara, CA, USA) in accordance with the manufacturer's instructions on the RNA 6000 Nano Labchip.

2.10. Quantitative real-time PCR

Primers and TaqMan fluorogenic probes specific for Atlantic cod Steroidogenic acute regulatory (*star*) protein mRNA were designed with Primer express software (Applied Biosystems, Oslo, Norway), according to the manufacturer's guidelines. The protocols for quantitative real-time PCR assays for Atlantic cod *20bhsd, cyp19a1a*, and for the endogenous control *18s* rRNA have been described previously (Mittelholzer et al., 2007). Sequences for all primers and probes are given in Supplement S3. All PCR assays were performed in duplicate, using 96-well optical plates on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Oslo, Norway) using default settings.

For each 25 μl PCR reaction cDNA was mixed with 200 nM fluorogenic probe, 900 nM sense primer, 900 nM antisense primer in 1xTaqman Fast Universal PCR Master Mix (Applied Biosystems, Oslo). For each PCR plate, no-template controls were run for each gene. The ΔΔCt method was used to calculate relative gene expression levels, as described in detail by Bogerd et al. (2001). Expression data was then presented as fold change compared to the mean values of ovarian mRNA levels in previtellogenic follicles in the 0.5% group in August, where mean Igs were lowest.

2.11. Data analysis

Data on steroid and VTG concentrations and gene transcript levels relative to ovarian maturation, were analysed using one-way ANOVA on specific days and stages. In cases where groups were not normally distributed, Kruskal-Wallis and post-test Dunn's analysis were used. Correlations were analysed using Product-Moment and Partial Correlation.

Where no other information is given, differences and effects are considered significant at $p < 0.05$.

3. Results

3.1. ARA and EPA:ARA ratio in plasma, ovaries and liver

3.1.1. Plasma

The relative ARA content and the EPA:ARA ratio in plasma were dose-dependent, and all groups were different from each other at all sampling points ($p < 4 * 10^{-3}$, Fig. 1a). The average content of ARA was lower in January than in September and May ($p = 10^{-4}$), while the average EPA:ARA ratio increased from 4.7 to 5.0 between September and January ($p = 0.04$) and decreased to 3.8 in May ($p = 10^{-4}$).

3.1.2. Ovaries

There was a clear increase in relative ARA content in the ovaries in response to increasing ARA in the diets at all sampling points after start of the experiment (all groups differed from each other, $p < 0.01$, Fig. 1b). ARA content decreased from September until January and increased again to a level higher than in September in spent ovaries in May ($p < 10^{-3}$).

The ratio of EPA:ARA in the ovaries reflected the diet, and was negatively correlated to dietary ARA content ($r^2 = 0.76$). Overall, the ratio was approximately twice as high in January, when most fish were in mid- or late vitellogenesis as in September or May, when the females were previtellogenic or spent ($p = 10^{-4}$). The sum of all fatty acids, measured as an estimation of lipid content, was not affected by the diet, but was more than 2.5-fold higher in January than in September and May ($p = 10^{-4}$).

3.1.3. Liver

There was a clear increase in liver ARA content in response to increasing dietary ARA levels at all sampling points after start of the experiment (Fig. 1c). In January and May, all groups were different ($p < 2 * 10^{-3}$), while in September fish fed 4% ARA were different from all other groups and fish fed 2% ARA was different from fish fed 0.5% ARA ($p < 0.02$). The liver EPA:ARA ratio showed a similar, but inverse, variation as liver ARA concentration. Liver ARA was slightly lower in May than in January ($P = 0.04$), while the level in September was intermediate.

Relative EPA and DHA contents in plasma, ovary and liver are presented in Supplement S4.

3.2. Ovarian development

Relative and potential fecundity did not vary significantly between groups (Table 2). The individuals with highest potential fecundity were found in the 2% and 4% groups, with 2010 and 2250 oocytes·g^{-1} and a potential fecundity of $10.9 \cdot 10^6$ and $11.0 \cdot 10^6$ eggs. female^{-1}, respectively.

No significant differences were observed in gonadosomatic index (I_G) between the four diet groups (data not shown). I_G was typically between 1 and 3% from June to October, when ovaries contained previtellogenic oocytes. During vitellogenesis, I_G increased from approximately 5% in early vitellogenic fish, to 10–15% in mid-vitellogenesis and 25–30% in late vitellogenesis. Maximum I_Gs between 40 and 45% were recorded in February and March, during the peak spawning period. The spawning period lasted from late January to mid-April. In May, when all fish were spent, average I_G was between 3% and 5%.

Between July and September, ovaries from females in all groups were mostly in the previtellogenic (PV) and spent (S) stages (Fig. 2). In October, around 90% of females fed 0.5% and 1% ARA had entered vitellogenesis and had ovaries in early vitellogenesis (stage VI). In the 2% group, 67% of the females had early vitellogenic ovaries, while 40% of the females in the 4% group had entered this stage. All sampled fish had entered vitellogenesis in November. In the groups fed 0,5% and 1% ARA, 86 and 83% had ovaries in early vitellogenesis, while 14 and 17% had mid-vitellogenic ovaries, respectively. A higher proportion of mid-vitellogenic females was apparent in the groups fed 2% and 4% ARA, with 26% and 23% of ovaries in mid-vitellogenesis, respectively. In December, the majority of females in all groups had mid-vitellogenic ovaries, with a low percentage of fish still in early vitellogenesis. The first females with late vitellogenic ovaries were observed in January, consisting of between 37 and 75% of the sampled individuals. In addition, 27% of the females fed 1% ARA were spawning in January. This group also contained the lowest proportion of late vitellogenic ovaries in January. In February and March, most of the sampled females were spawning, with less than 20% of fish with ovaries in mid- or late vitellogenesis. All fish were characterized as spent in May.

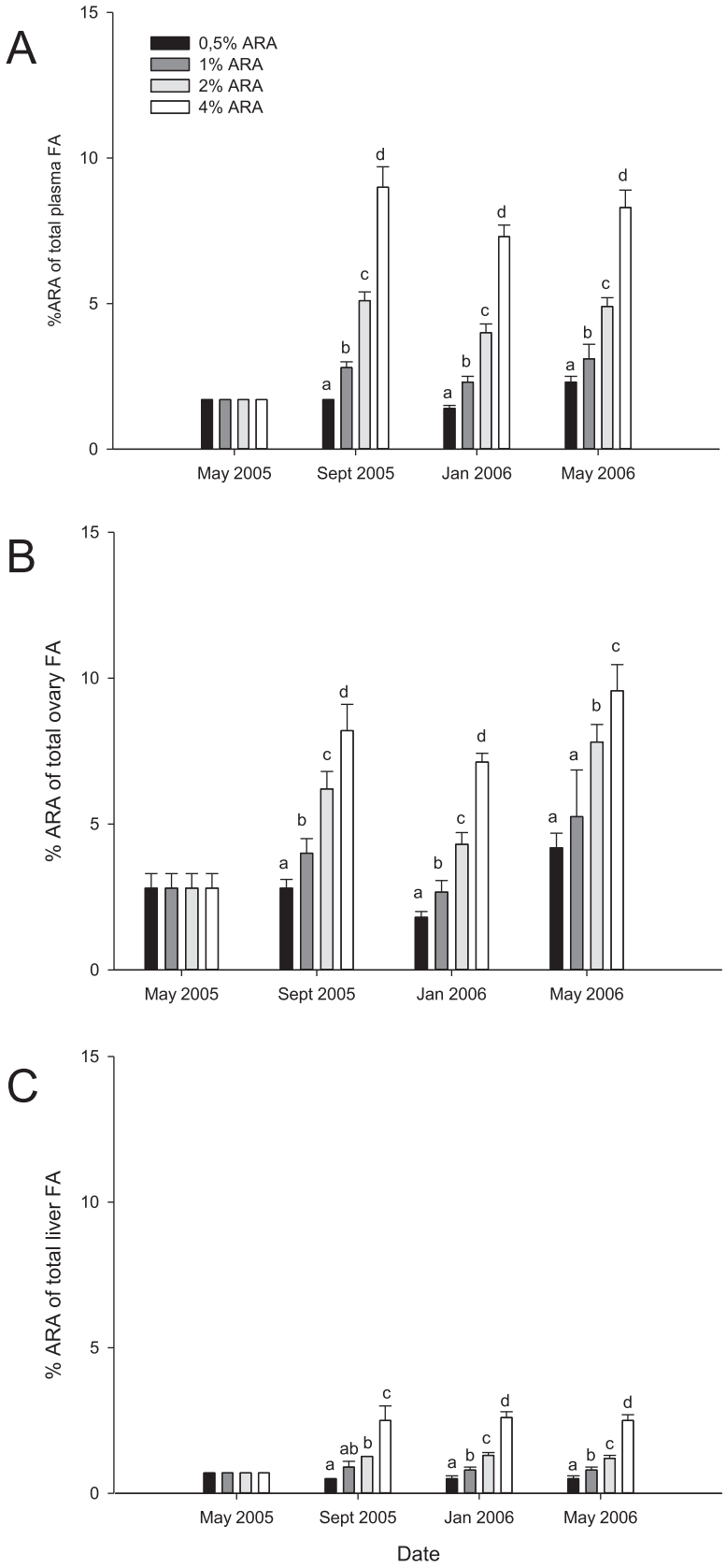

Fig. 1. Relative content of ARA in plasma (a), ovaries (b) and liver (c) of female Atlantic cod fed diets with four different concentrations of ARA: 0.5%, 1%, 2% and 4% of total fatty acids, respectively. Bars show mean content ± SEM. N = 5–8 individuals. diet^{-1}.

Table 2
Potential fecundity, potential relative fecundity, length and weight of female Atlantic cod fed graded levels of ARA. Samples taken in the period from November to January.

Diet	Measure	Mean	N	SD	Min	Max	Lower 95% CI	Upper 95% CI
0.5%	Fecundity (millions)	6.5	36	1.7	2.8	9.7	5.9	7.1
	Relative fecundity (n/g)	1337	36	275	740	1969	1244	1431
	Length (mm)	667	36	47	480	760	651	683
	Weight (g)	4891	36	969	2306	6365	4563	5219
1%	Fecundity (millions)	6.5	27	1.8	2.8	10.6	5.8	7.3
	Relative fecundity (n/g)	1309	27	247	682	1832	1211	1407
	Length (mm)	676	27	39	580	770	661	692
	Weight (g)	4992	27	1085	2582	7645	4563	5421
2%	Fecundity (millions)	6.5	37	1.9	3.0	10.9	5.9	7.1
	Relative fecundity (n/g)	1333	37	298	846	2010	1234	1433
	Length (mm)	659	37	54	540	780	641	678
	Weight (g)	4876	37	1015	2536	7310	4537	5214
4%	Fecundity (millions)	5.9	31	1.7	3.3	11.0	5.3	6.5
	Relative fecundity (n/g)	1308	31	272	706	2250	1208	1408
	Length (mm)	651	31	52	490	720	632	670
	Weight (g)	4509	31	902	2609	6140	4178	4840

3.3. Plasma steroids and vitellogenin

3.3.1. Annual profiles

Plasma testosterone (T) concentrations (Fig. 3a) were low or undetectable during the summer months, increased from August to September ($p < 10^{-4}$) and reached peak levels in September in fish fed 2 and 4% ARA, and in October in fish fed 0.5 and 1% ARA. The concentrations then decreased to similar levels in all groups in November ($p < 0.01$) and remained elevated compared to summer levels until February ($p < 10^{-4}$). In March through to May, T levels were similar to the levels found during the summer. In October, the T concentration in fish fed 1% ARA was 5.78 ± 1.01 ng·ml^{-1}, significantly higher than in the 2% and 4% groups ($p < 0.03$), and similar to the 0.5% group (5.23 ± 0.81 ng·ml^{-1}), which was different from T in fish fed 4% ARA ($p = 0.02$). In September, T levels in fish fed 2 and 4% ARA reached 3.93 ± 0.35 ng·ml^{-1} and 3.96 ± 1.17 ng·ml^{-1}, respectively, and were significantly higher than plasma T levels in the 0.5% group ($p < 0.05$), while T in the 1% ARA group was intermediate. In the 4% ARA group, an apparent T peak of 4.89 ± 2.13 ng·ml^{-1} was present in late January. However, the individual variation was high in this group, and the mean concentration was not significantly different from that in the other diet groups.

Plasma estradiol-17β (E2) concentrations were below 3 ng·ml^{-1} from June until September, were between 3 and 8 ng·ml^{-1} in November and December and then increased rapidly in all groups ($p < 5 * 10^{-4}$) until January (Fig. 3b). In the 1 and 2% ARA groups, plasma E2 concentrations peaked in January at 19.15 ± 2.62 ng·ml^{-1}, and 22.19 ± 4.52 ng·ml^{-1}, respectively. Plasma E2 concentrations in the 4% ARA group peaked in February at 26.51 ± 2.33 ng·ml^{-1}, while in the 0.5% ARA group, concentrations were high at all sampling points from January until March at max 19 ± 11 ng·ml^{-1}. The concentrations of E2 in plasma were still elevated in all groups in March, and declined to below 1 ng·ml^{-1} in May ($p < 5 * 10^{-4}$). E2 in fish fed 4% ARA was significantly higher than in all the other groups in February ($p < 0.03$). No other significant differences in E2 between groups at single sampling points were registered.

Vitellogenin (VTG) concentrations were below 5 mg·ml^{-1} in all groups from June through September, after which concentrations increased ($p < 5 * 10^{-4}$, Fig. 3c). Fish fed 1, 2 and 4% ARA reached peak VTG concentrations in November, at 22.05 ± 2.62, 25.12 ± 3.05 and 23.59 ± 2.18 mg·ml^{-1}, respectively. In the 0.5% group, VTG reached its highest concentration in December at 21.83 ± 6.73 mg·ml^{-1}. VTG concentrations remained elevated at above 7 mg·ml^{-1} until March in all groups and then decreased to below 5 mg·ml^{-1} in May ($p < 0.001$, Fig. 3c). In November, VTG was lower in fish fed 0.5% than in those fed 2 and 4% ARA ($p < 0.02$), while fish fed 1% ARA were intermediate. In December VTG was higher in fish fed 0.5% than in those fed 2 and 4%ARA ($p < 0.02$), and again fish fed 1% ARA was intermediate (Fig. 3c). There was a weak, but significant correlation between plasma E2 and VTG ($r^2 = 0.204$; $P \leq 0.05$). However, since VTG attained peak levels one to two months earlier than E2, the highest (>20 mg·ml^{-1}) VTG levels were generally accompanied by low levels of E2.

3.3.2. Changes in relation to ovarian stage

An overview of significant differences in plasma steroid and VTG concentrations between ovarian developmental stages within the diet groups is shown in Supplement S5.

Plasma testosterone (T) concentrations varied between ovarian stages ($p = 10^{-4}$) (Supplement S5). In all groups except fish fed 4% ARA, highest T concentrations during maturation were observed at the early vitellogenic stage. At this stage, T was significantly higher in the 0.5% group than in the 4% group (Fig. 4a). This was the only significant difference observed between diet groups. In the 4% group, plasma T concentrations appeared to be highest at late vitellogenesis, but this was not significant, most likely due to high individual variation. Plasma T concentrations were lowest in spent fish.

Estradiol-17β (E2) increased through ovarian maturation (Supplement S5). The highest concentrations were found at late vitellogenesis and spawning, i.e. stages VIII and S, while the lowest E2 concentrations were detected in previtellogenic and spent fish; stages PV and SP. Significant differences between dietary groups were only present in spawning fish, where the 4% group had higher E2 concentrations than the 1% and 2% groups and the 0.5% ARA group was intermediate (Fig. 4b).

Plasma VTG concentrations were highest in early and mid-stages of vitellogenesis, stages VI and VII, and lowest in previtellogenic and spent fish (Supplement S5). At stage VI, VTG concentrations were significantly higher in females fed 2% and 4% ARA, than in females fed 0.5% ARA (Fig. 4c). This was the only stage where significant differences in plasma VTG were found.

3.4. Gene expression

An overview of significant differences in gene transcript levels between ovarian developmental stages within the diet groups is shown in Supplement S6.

Transcript levels of *star* increased in late vitellogenic females and were significantly higher in spawning females than in individuals with pre- early- or midvitellogenic ovaries from all diet groups

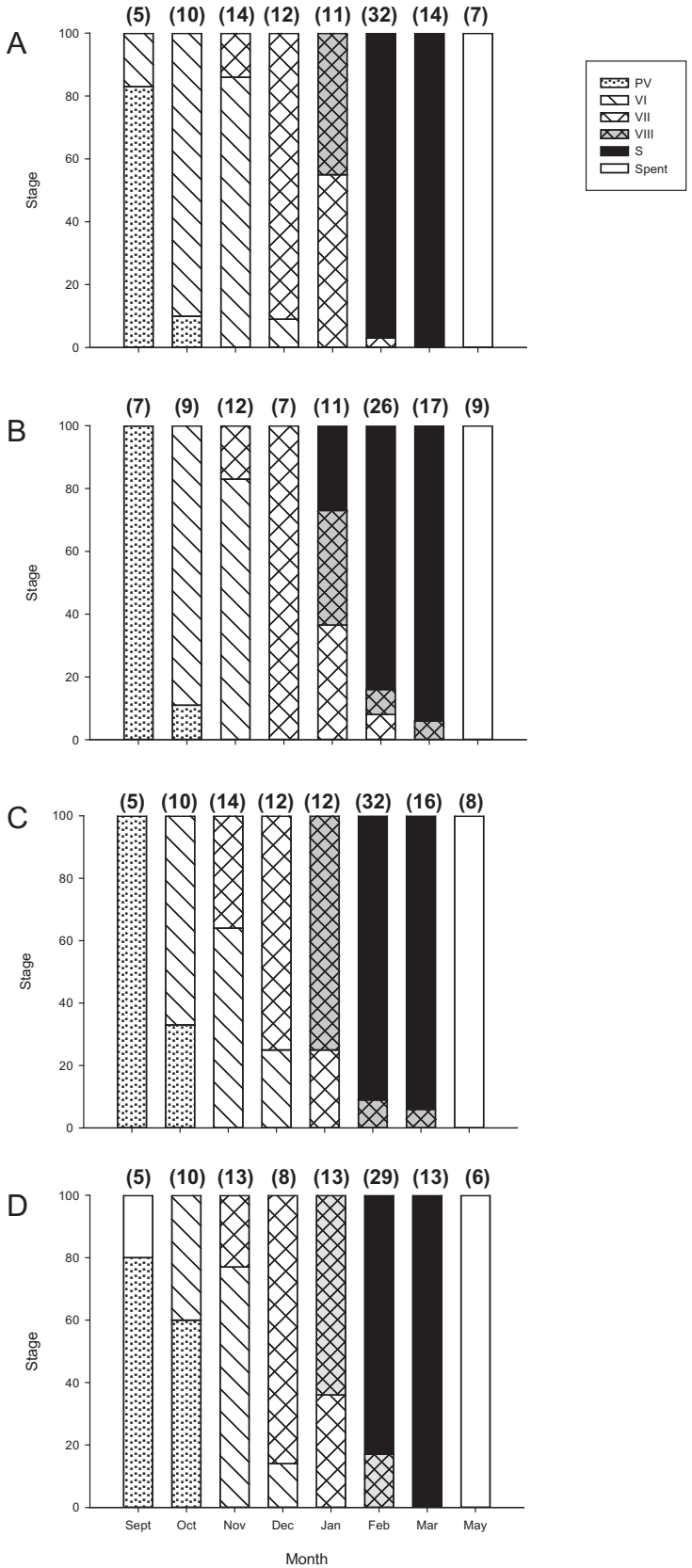

Fig. 2. Changes in relative amounts of oocytes at different stages of development over time. (a) 0.5% ARA (n = , b) 1% ARA, (c) 2% ARA and (d) 4% ARA. PV = previtellogenic; VI = early vitellogenic; VII = mid-vitellogenic; VIII = late/post-vitellogenic; S = Spawning; Sp = spent. Numbers in parentheses above bars represent n at each sampling.

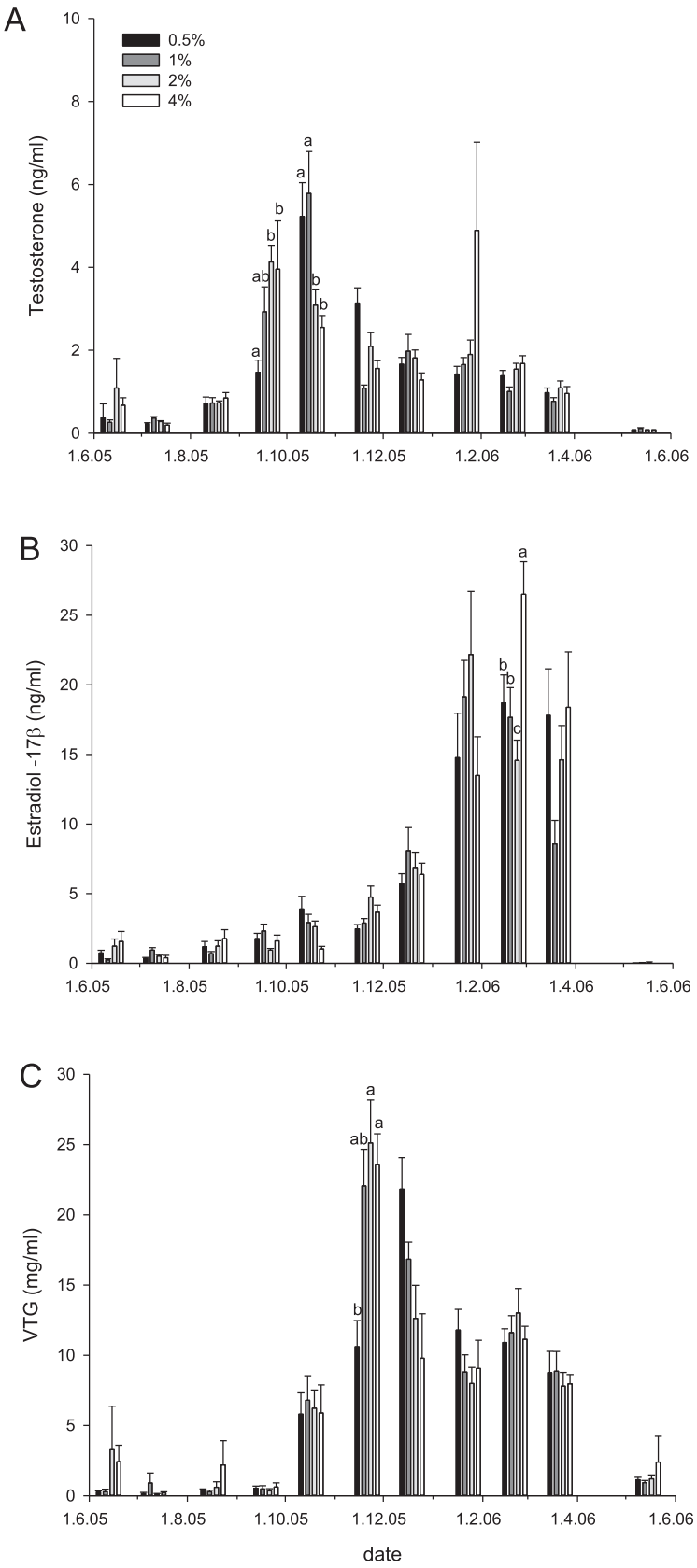

Fig. 3. Annual variations in plasma concentrations of (a) testosterone, (b) estradiol-17β, and (c) vitellogenin in female Atlantic cod fed diets with four different concentrations of ARA. Different letters on top of the bars mark significant differences between diets at the same sampling time point. Bars show mean concentration ± SEM. n = 5–9 (June to September); 8–15 (October–January; March–May); 26–32 (February).

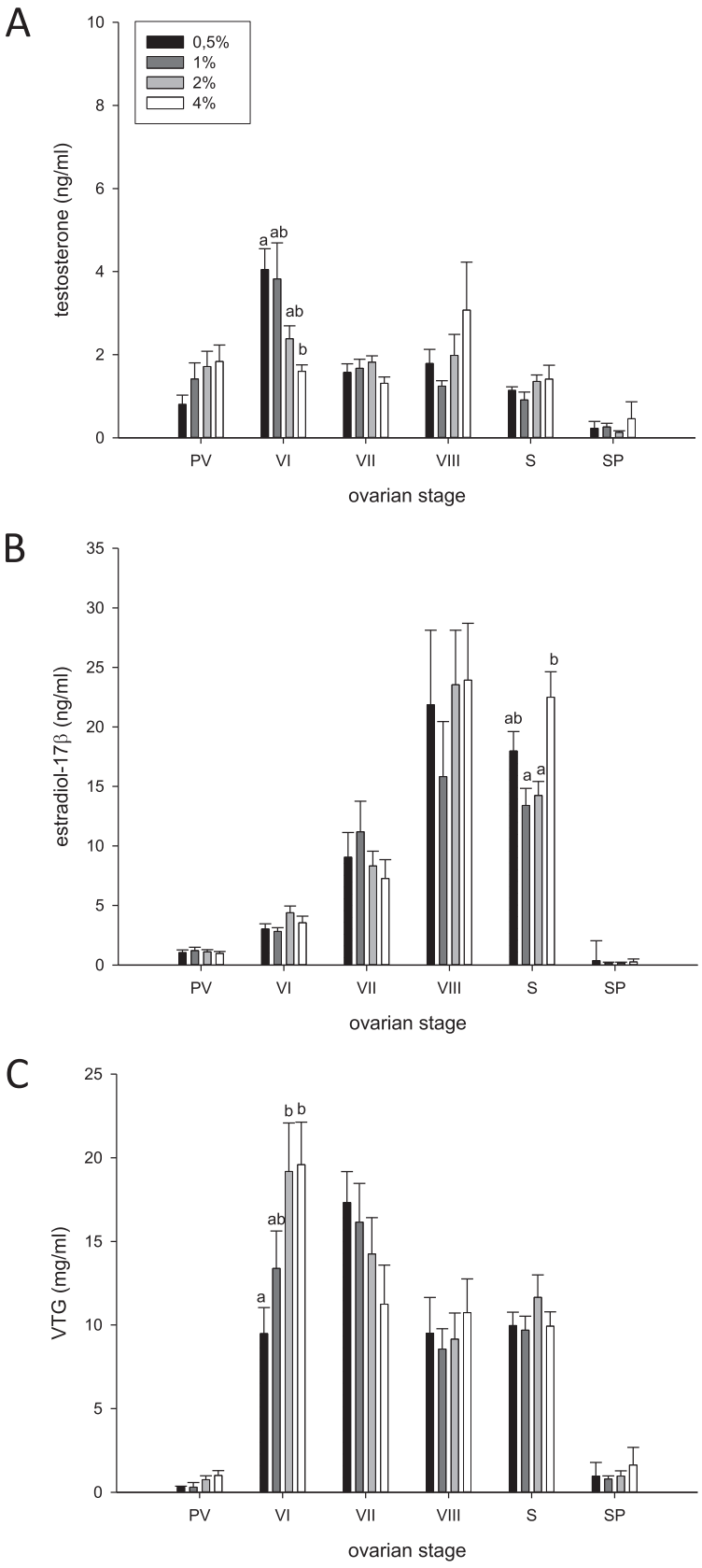

Fig. 4. Plasma concentrations of (a) testosterone, (b) estradiol-17β and (c) vitellogenin at different stages of ovarian development in female Atlantic cod fed diets with four different concentrations of ARA. Different letters on top of the bars mark significant differences between diets at the same sampling time point. Bars show mean concentration ± SEM. PV = previtellogenic (n = 12–15); VI = early vitellogenic (n = 11–20); VII = mid-vitellogenic (n = 11–19); VIII = late/post-vitellogenic (n = 7–13 except for 0.5% ARA where n = 5); S = Spawning (n = 35–45 except for testosterone and 4% ARA, where n = 25); Sp = spent (n = 6–10 except for VTG at 4% ARA where n = 4).

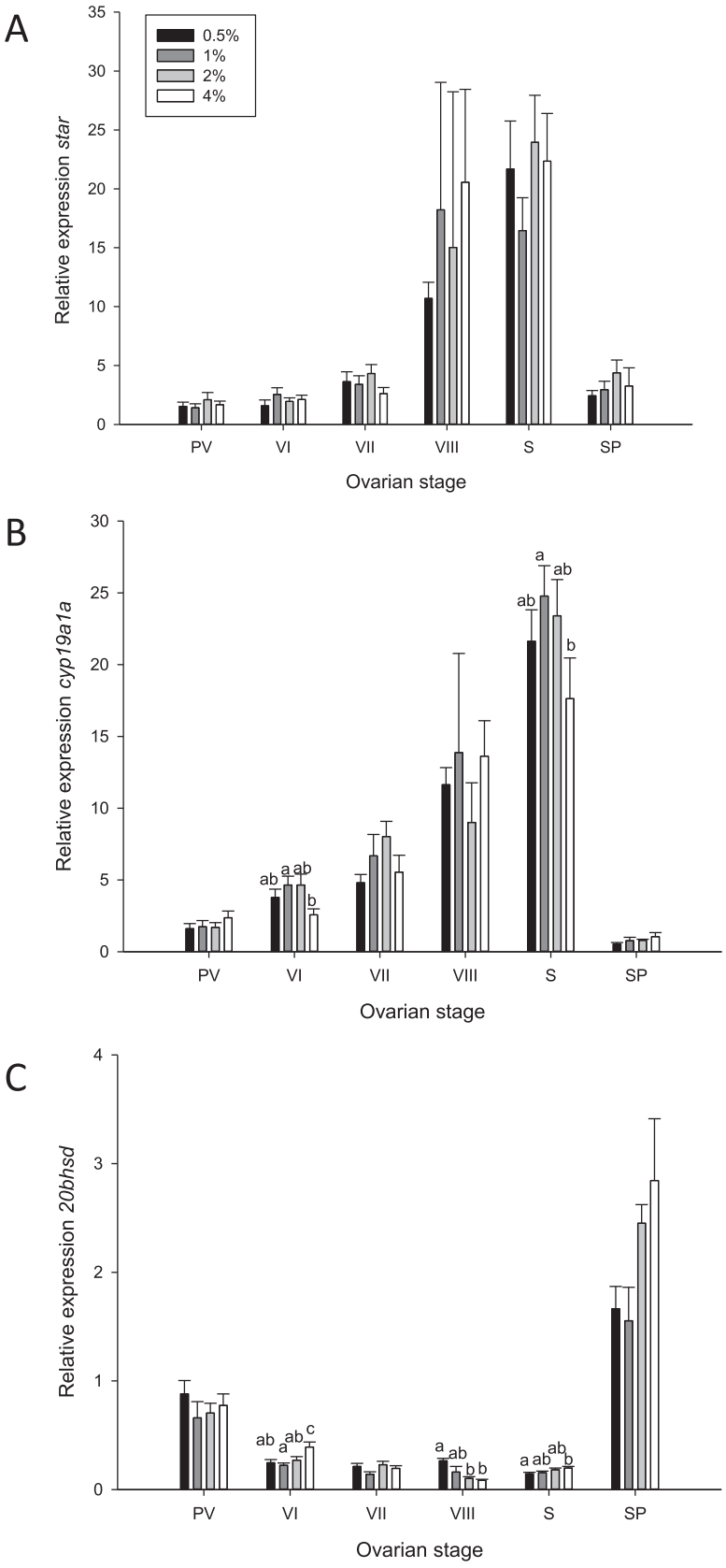

Fig. 5. Ovarian gene transcript levels of (a) *star*, (b) *cyp19a1a* and (c) *20bhsd* relative to *18S* at different stages of ovarian development in female Atlantic cod fed diets with four different concentrations of ARA. Different letters on top of the bars mark significant differences between diets at the same sampling time point. Bars show mean transcript levels ± SEM. PV = previtellogenic (n = 11–15 except for *star*, where n = 7–11); VI = early vitellogenic (n = 15–22 except for *star* at 4% ARA where n = 10)); VII = mid-vitellogenic (n = 7–13 except for 0.5% ARA where n = 3–5); VIII = late/post-vitellogenic (n = 7–13 except for 0.5% ARA where n = 5); S = Spawning (n = 32–44); Sp = spent (n = 7–10 except for *20bhsd* at 2% and 4% ARA where n = 4 and 5, respectively).

(Supplement S6). *Star* expression returned to low levels in spent fish. No significant differences in star mRNA levels were found between the four diet groups at any stage (Fig. 5a).

Expression of *cyp19a1a* increased through vitellogenesis ($p < 0.0001$), and mRNA levels were highest at spawning in all groups except females fed 4% ARA, where no significant differences were found between spawning females and females that were in mid- or late vitellogenesis, (Supplement S6). *cyp19a1a* transcript levels were lower in the 4% group at early vitellogenesis and spawning than in females fed 1% ARA, while fish fed 0.5% and 2% ARA were intermediate (Fig. 5b). Overall, the lowest levels of *cyp19a1a* mRNA were found in spent females.

Transcript levels of *20bhsd* were twofold higher in previtellogenic fish than in vitellogenic and spawning fish and increased on average 4–6-fold between spawning and spent females (Supplement S6). In early vitellogenic, as well as in spawning females, *20bhsd* mRNA levels were slightly, but significantly, higher in the 4% group than in the 0.5% and 1% diet groups (Fig. 5c). At late vitellogenesis, *20bhsd* transcripts were more abundant in the 0.5% group than in the groups fed 2% or 4% ARA.

3.5. Correlation between Ig, steroid and VTG concentrations, star, cyp19a1a and 20bhsd mRNA levels

A correlation matrix is shown in Table 3.

Overall, Ig, plasma E2 and VTG concentrations and ovarian *cyp19a1a* gene transcript levels were positively correlated. E2 was positively correlated to *star* and *20bhsd*. *Star* also showed positive correlations with *cyp19a1a* and *20bhsd*. T showed weak negative correlations with E2 and *star*.

4. Discussion

Dietary arachidonic acid (ARA) had significant effects on the endocrine regulation of ovarian development in Atlantic cod. Effects of ARA on fecundity and egg and larval quality in teleost fish, including cod, have been well documented (e.g. Bruce et al., 1999; Furuita et al., 2003; Lund et al., 2008; Mazorra et al., 2003; Pickova et al., 2007, 1997; Røjbek et al., 2014; Salze et al., 2005). Furthermore, a number of studies have documented *in vitro* effects of ARA and other fatty acids on steroidogenesis and follicular maturation in fish (Knight and Van der Kraak, 2015; Lister and Van der Kraak, 2008, 2009; Mercure and Van Der Kraak, 1995, 1996; Patino et al., 2003; Sorbera et al., 2001; Van Der Kraak and Chang, 1990; Wade et al., 1994). In contrast, few studies address the influence *in vivo* of dietary ARA on reproductive physiology and endocrinology (Norambuena et al., 2013a,b). This is to our knowledge the first documentation of physiological and endocrine effects *in vivo* on reproductive development in a female coldwater marine teleost fed experimental diets with varying proportion of ARA in relation to total fatty acids.

ARA concentration in gonad, liver and plasma increased in response to dietary ARA as expected. Plasma and ovarian ARA concentrations were very similar, while the liver accumulated less ARA than the ovaries. This is in line with results from Senegalese sole fed diets with six different ARA concentrations, where ARA accumulated in the ovaries and female livers, but only at the highest dietary level compared to the lowest (6% vs 0.7%) (Norambuena et al., 2013b). In the present study, the decrease in average ovary ARA concentration from September (mainly pre-vitellogenic) until January (pre-spawning) and the increase from January until May when the ovary was spent, indicate that egg yolk accumulated less ARA than ovarian tissue. ARA therefore seems to have been actively incorporated into ovarian tissue, possibly due to a special function in steroid synthesis as documented above.

Energy investment in ovarian growth, as monitored by Ig and potential fecundity, did not appear to be affected by the proportion of dietary ARA. Overall, female cod held as broodstock in captivity invest more energy in reproductive development than wild females. While average fecundity values in the present study varied between 1200 and 1300 oocytes·g fish^{-1}, typical average values for wild cod are between 400 and 600 (Thorsen et al., 2006, 2010). However, Kjesbu et al. (1991) found average values of approximately 1200 in groups of captive cod, which is similar to our current findings.

Ovarian development in cod has been described by histology and oocyte size frequency distribution in several previous publications (e.g. Dahle et al., 2003; Kjesbu et al., 1996a). In the present study, we chose to classify ovarian development according to size of leading oocyte cohort as a measure of ovarian stage (Thorsen et al., 2006). When using this method, some differences were apparent between the experimental groups. Females fed 0.5% and 1% ARA appeared to enter vitellogenesis earlier than females fed higher ARA levels, as a higher percentage of females in those groups had oocytes in stage VI, early vitellogenesis, in October. Vitellogenesis proceeded in all groups through November and December, with a gradual increase in mid-vitellogenicovaries. The first spawning individuals were found in January, one month earlier in females fed 1% ARA than in the other groups. A few individuals fed 0.5% and 1% ARA remained in mid-vitellogenesis in February, while most of the sampled fish were spawning. Taken together, these results may indicate an effect of ARA on the timing of vitellogenesis and spawning, but further experimental work is needed in order to confirm or reject this hypothesis.

Annual plasma profiles of VTG have not previously been documented in Atlantic cod. Plasma VTG concentrations increased from October, and were highest in November and December. However, as I_Gs were still low, and oocytes correspondingly small, VTG uptake rates and plasma clearance were lower during this period than at later stages of development when VTG uptake was going at maximum rate. The observed "peaks" are therefore likely to reflect low VTG uptake rather than high rates of synthesis. Plasma VTG levels remained elevated until March, through the spawning period. This has also been documented in other periodic (batch) spawners, such as Senegalese sole, Atlantic halibut and European sea bass (Guzman et al., 2008; Methven et al., 1992; Navas et al., 1998) and may be due to ongoing vitellogenesis in some clutches of oocytes while others are recruited into final maturation.

Table 3
Correlation matrix showing correlations between Ig, plasma T, plasma E2, plasma VTG, and gene transcript levels of *cyp19a1a, 20bhsd* and *star*. Numbers in bold italics represent significant correlations.

	Ig	T	E2	VTG	*cyp19a1a*	*20bhsd*	*star*
Ig	1,0000	***−0,1234***	***0,6366***	***0,2255***	***0,6617***	0,0568	***0,4046***
Testosterone (T; ng/ml)	***−0,1234***	1,0000	−0,0041	0,0269	−0,0626	0,0342	***−0,1091***
Estradiol (E2; ng/ml)	***0,6366***	−0,0041	1,0000	***0,2036***	***0,4849***	***0,1955***	***0,2695***
VTG (μg/ml)	***0,2255***	0,0269	***0,2036***	1,0000	***0,1786***	−0,0005	0,0761
cyp19a1a	***0,6617***	−0,0626	***0,4849***	***0,1786***	1,0000	0,0543	***0,4420***
20bhsd	0,0568	0,0342	***0,1955***	−0,0005	0,0543	1,0000	***0,1422***
star	***0,4046***	***−0,1091***	***0,2695***	0,0761	***0,4420***	***0,1422***	1,0000

Plasma steroid levels were correlated with ovarian status, in accordance with what has been reported previously both in Atlantic cod (Dahle et al., 2003; Norberg et al., 2004; Skjaeraasen et al., 2004) and other teleost fish (reviewed by Lubzens et al. (2010)). Plasma T levels were highest during onset of vitellogenesis, while plasma E2 increased steadily until late vitellogenesis, E2 concentrations remained elevated in spawning females. Individual variations in E2 were high during spawning, reflecting the simultaneous presence of vitellogenic and maturing oocytes that is found in cod, as well as in other multiple group-synchronous (batch) spawners (e.g. Bjornsson et al., 1998; Garcia-Lopez et al., 2006, 2007; Guzman et al., 2008; Karimi et al., 2014; Kjesbu, 1989, 1994; Methven et al., 1992; Navas et al., 1998; Norambuena et al., 2013a; Sun and Pankhurst, 2004; reviewed by Pankhurst (2008)).

Interestingly, an apparent desynchronization in late vitellogenic (VIII) individuals may have occurred in the group fed the diet with the highest ARA content. This was reflected especially in plasma T levels, where an apparent "peak" resulting from high individual variation in plasma T was present. The high T levels were seen in a few individuals that had reached the late vitellogenic stage, but none in mid-vitellogenesis in this group. At the same time, E2 and VTG concentrations remained relatively low. A possible explanation is that these individuals were very close to ovulation. High concentrations of T and low concentrations of E2 are present at this stage in salmonids, where vitellogenesis is clearly separated in time from final maturation and spawning (e.g. Norberg et al., 1989; Andersson et al., 2013). Normally, Atlantic cod exhibit nocturnal spawning behaviour, with greatest activity around dawn and dusk (Kjesbu, 1989), as seen in many species with pelagic eggs (Ferraro, 1980). If the high proportion of ARA had a negative effect on the synchronization of spawning, it may also be possible that the diurnal reproductive rhythms were affected, and a steroid pattern that would mostly be present before dawn was seen in fish sampled later in the day. This is, however, highly speculative and the number of individuals with high T concentration was low.

Plasma E2 concentrations were significantly higher during spawning in the 4% ARA group. In the European sea bass, feeding female broodstock with commercial growout diets, resulted in altered plasma profiles of sex steroids and VTG (Cerda et al., 1995; Navas et al., 1998) compared to females fed a "natural" diet of trashfish. In the individuals that received commercial diets, reproductive performance was impaired even after addition of fish oil enriched with n-3 fatty acids. In a subsequent study on sea bass broodstock nutrition, addition of tuna orbital oil, which is high in DHA, and in ARA in particular, to the diet significantly improved egg survival and hatching (Bruce et al., 1999). Bogevik et al. (2014) found that feeding male sea bass a diet with a high content of plant oil resulted in delayed sexual maturation and suppression of maturation-related gene expression, compared to fish fed a diet based on marine oil. While plant oils can contain high amounts of C18 n-6 fatty acids such as linoleic acid, fish cannot elongate them to ARA which is usually supplied through marine protein and marine oils in the feed. In Senegalese sole, high dietary ARA levels were correlated with increased plasma sex steroid concentrations in males, but not in females (Norambuena et al., 2013a). Further, in tongue sole (*Cynoglossus stenolaevis*) fed high levels of dietary ARA, plasma E2 levels were significantly lower in both immature and mature females, while mature males had higher testosterone concentrations (Xu et al., 2017). This suggests that there may be both gender- and species-specific differences in the physiological effects of ARA on reproductive endocrinology.

Transcript levels of *star* were positively correlated with Ig and plasma E2 concentrations and were most abundant in late vitellogenic and spawning female cod, when rates of steroid synthesis and plasma concentrations are expected to be highest. This is consistent with previously published work on cod (Goetz et al., 2004) as well as other species (Bobe et al., 2004; Kusakabe et al., 2002; Nakamura et al., 2005; Rocha et al., 2009).

We did not find any significant differences in *star* expression between diets, even though the 0.5% group appeared to have lower abundance of *star* transcripts in late vitellogenic ovaries. This is in apparent contrast to tongue sole, where relative *star* expression increased in immature females fed 5%, but not 15%, ARA. The individual variation in our study was, however, too high to reveal any significant effects of dietary ARA content on *star* transcript levels. It should also be noted that the relative ARA content in the diets given to cod ranged from 0.5% to 4%, and that species differences in optimal ARA concentrations and effects make direct comparisons difficult. *In vitro* studies in mammals have shown a direct stimulatory effect of ARA on *star* expression, at the level of transcription (Wang et al., 2000; Wathes et al., 2007; cf. Stocco et al., 2005). Even though we could not detect any significant effects of ARA *in vivo* in Atlantic cod, further studies are needed where ovarian follicles in different stages of maturation are separated at a higher resolution, in order to elucidate whether ARA can regulate steroidogenesis through StAR in this species.

cyp19a1a transcript levels increased steadily through ovarian development and were highest at late vitellogenesis and spawning, though only significantly so in spawning females. Further, ovarian *cyp19a1a* transcript levels showed a clear correlation with *Ig* as well as plasma E2 and VTG concentrations. Numerous previous studies have described high and increasing levels of expression of *cyp19a1a* through vitellogenesis, in cod (Breton et al., 2012), as well as other teleost species (e.g. Kumar et al., 2000; Nakamura et al., 2005; Rocha et al., 2009). In apparent contrast to these studies, we found the highest *cyp19a1a* transcript levels in spawning female cod. However, individual cod females can spawn 15–20 consecutive egg batches at 48–60 h intervals during their spawning season (Kjesbu, 1989; Kjesbu et al., 1990) and large numbers of vitellogenic oocytes will be present in their ovaries, especially at the start of spawning. The seemingly unusual and high levels of *cyp19a1a* transcripts were also reflected in high plasma E2 concentrations in spawning cod.

Some differences were apparent between the four diets. *cyp19a1a* transcripts were less abundant in females given 4% ARA in early vitellogenesis and during spawning. This is in agreement with results reported in tongue sole where dietary ARA suppressed relative expression of *cyp19a1a* in both immature and mature females (Xu et al., 2017). However, E2 concentrations were higher during spawning in plasma of females in the 4% group, indicating a negative feedback of high plasma E2 on *cyp19a1a* expression and subsequent P450-aromatase activity. Addition of ARA to the broodstock diet appears to have a species-specific optimum level (Bell and Sargent, 2003) and higher concentrations of ARA, or lower EPA:ARA ratio can result in lower realized fecundity, although the optimal ARA concentration has not been determined for cod. A diet containing 4% ARA of total FA may be above the optimum supplementation level. Females in this group also had lower realized as well as relative fecundity than females from the other diet groups, as seen in a spawning experiment run in parallel with, and using fish taken from the same experimental groups, as the present study (Norberg et al., 2008).

The overall pattern of change in *20bhsd* expression through the maturation cycle revealed some interesting features. *20bhsd* transcript levels were elevated twofold in previtellogenic ovaries compared to vitellogenic and maturing ovaries. (Miura et al., 2007) proposed that progestins have a role together with estrogens in regulation of early stages of oogenesis in fish, which would require an elevated activity of 20βHSD and other key steroidogenic enzymes. High transcript levels of *20bhsd* have been found previously in previtellogenic ovaries from immature Atlantic cod

(Kortner et al., 2009), as well as in early developing ovaries of gilthead sebream (*Sparus aurata*) (Zapater et al., 2012). Moreover, Zapater et al. (2012) found that Follicle-stimulating hormone (Fsh) increased *20bhsd* mRNA levels and stimulated production of 17,20β-P *in vitro* in primary ovarian follicles from gilthead seabream. Although the role for progestins in early ovarian development is not clear, it has been suggested to act directly to trigger the first meiotic division in primary oocytes of Japanese huchen (*Hucho perryi*) and common carp (*Cyprinus carpio*) (Miura et al., 2007) or, alternatively, to regulate gene transcription in early ovarian follicles (Zapater et al., 2012).

The low transcript levels of *20bhsd* during final maturation and spawning in cod is in apparent contrast to some previous studies, which showed increasing expression during final maturation and spawning in other teleost species such as ayu (*Plecoglossus altivelis*), tilapia and rainbow trout (Nakamura et al., 2005; Senthilkumaran et al., 2002, 2004; Tanaka et al., 2002). On the other hand, (Bobe et al., 2003, 2004) did not find any changes in *20bhsd* transcript levels in rainbow trout ovarian follicles during maturation and suggested involvement of other factors such as other enzymes or growth factors, and/or substrate availability. Wang and Ge (2002) found that *20bhsd* expression appeared to be constitutive and did not change across development stages in ovarian follicles from zebrafish, which are asynchronous batch spawners. Recently, Ijiri et al. (2017) identified another enzyme, which is similar to but not identical with 17β-hydroxysteroid dehydrogenase type 12 (HSD17β12), in granulosa cells of masu salmon during final oocyte maturation. These authors suggested that this enzyme (HSD17β12l), and not 20βHSD, is responsible for 17,20β-P production in masu salmon. Whether this is also the case for other teleosts remains to be investigated, especially in species such as the cod, where the MIS has been suggested to be 17,20β,21-P rather than 17,20β-P (Tveiten et al., 2010) .

Relative expression of *20bhsd* was highest in spent female cod, one to two months after completion of spawning. This is in agreement with results reported in other species such as rainbow trout, tilapia and ayu (Nakamura et al., 2005; Senthilkumaran et al., 2002; Tanaka et al., 2002), where highest levels of *20bhsd* transcripts were found in postovulatory follicles. Pinillos et al. (2002) and Antonopoulou et al. (2011) found that MIS plasma concentrations peaked two months after spawning in the tench (*Tinca tinca*) but, unlike the situation in huchen and carp (Miura et al., 2007), it was not correlated to onset of first meiosis. Unfortunately, we could not measure plasma MIS concentrations in the present study, but the high *20bhsd* transcript levels can be seen as an indirect indication that a similar profile as in the tench may be present.

Only small differences in *20bhsd* transcript levels were found between the diet groups. Abundance of *20bhsd* transcripts was higher in the 4% group than in the 0.5% group in early vitellogenic and spawning ovaries, at the same time as *cyp19a1a* transcript levels were lower. However, the significance of these findings is unclear as the differences were very small and any physiological effect, if present, can be expected to be subtle.

In conclusion, the present study has provided evidence that dietary ARA content influences the reproductive physiology of female Atlantic cod. Some shifts in the plasma profiles of E2 and T, as well as *cyp19a1a* transcript levels suggest an influence on steroidogenesis, but the precise mechanisms for a possible influence of ARA on endocrine regulation of reproductive development remain unclear. Future investigations should also address possible regulation at other steps in steroidogenesis, such as the actions of P450c17-I and P450c17-II that are important regulators of earlier steps in the synthesis of E2 and MIS, respectively. Knowledge on the regulatory mechanisms of ARA on reproduction is important in the design and development of broodstock diets for optimal fish health and gamete quality. Whether changes in distribution, fatty acid composition and abundance of prey species can also have an influence on fecundity, egg survival and recruitment of wild cod stocks in the future is an interesting possibility that merits further investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2017.05.020.

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